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Signature and Mechanism of the Epithelial-to-Mesenchymal Transition

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14. ABSTRACT

The Epithelial-to-Mesenchymal Transition (EMT) is a conserved developmental process that is thought to be reactivated during the metastasis of epithelial cancers such as breast cancer. This study seeks to identify genes commonly regulated in the EMT, and identify key regulators of the process. The transcription factor Zeb1 was identified as a strong and rapid inducer of the EMT. A microarray study of the genes directly downstream of Zeb1 would be very useful in studying its mechanism of action. A Zeb1-inducible expression system was deemed highly desirable for this experiment. A Tamoxifen-responsive system was created, but failed to achieve the desired characteristics to be of any utility. A Doxycycline-responsive system was subsequently created which had the desired characteristics of high inducibility coupled with low leakiness. Single-cell clones expressing the desired constructs were obtained and further optimization of the system is underway to maximize synchronicity of the cells, and thusly the signal obtainable by the microarray experiment.

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Introduction

Breast cancer is one of the leading causes of death in women of the developed world. Mortality results not from the primary tumor, but from complications arising from metastases that have spread to vital organs. The study of breast cancer progression and metastasis is therefore key to developing effective treatments. The Epithelial-to-Mesenchymal Transition (EMT) is a conserved embryonic developmental process where tightly bound, non-motile epithelial germ layer cells acquire the characteristics of mesenchymal cells, allowing them to migrate away from their original site(Hay, 1995). The EMT is thought to be aberrantly turned on during cancer metastasis, and may facilitate the dissemination of cancer cells to distant organs. The EMT is not well-defined at a molecular level, and it is not known which aspects of the EMT are important in metastasis. This study seeks to identify a list of genes that characterize the EMT, as well as key regulators within the list. A model genetic circuit of the EMT will be built and tested using the key regulators as anchor points. The long-term goal is to develop a molecular understanding of mechanisms critical to metastatic progression, which will guide strategies to curtail the spread of cancer.

Body

With regard to the training plan, the teaching component of the plan has been completed in May 2009. All meetings, symposia and conferences listed in the Statement of Work were attended.

With regard to the research plan:

1. Generate a core gene expression signature of the EMT using HMLE-derived cell lines

Using cell lines already available in the lab, microarray expression profiling was employed to generate a gene signature of approximately 1000 genes which were consistently up- or down-regulated in cells that had converted to a mesenchymal phenotype.

2. Use the core gene signature to find key components of the EMT regulatory circuitry

Attempts to draw a gene network solely from the expression profiling data were unsuccessful. Several well-established bioinformatics algorithms were employed, but did not yield strong candidates(Basso et al., 2005),(Subramanian et al., 2005). Correlating gene expression to genomic locations commonly amplified or deleted in cancers did not produce meaningful results. Comparing existing cancer metastasis gene signatures to the core signature did not yield any overlap over what could be expected by chance(van 't Veer et al., 2002). Eventually, comparison of the EMT signature to existing high quality microarray data from clinical samples of melanoma primary tumors versus melanoma metastases produced a significant overlap(Jaeger et al., 2007). An analysis of the promoters of the overlapping set of genes resulted in the identification of the transcription

factor Zeb1 as a likely mediator of many genes whose activities change upon transition to the mesenchymal state(Loots and Ovcharenko, 2004).

3. Test the effect and importance of candidate key components on the EMT in HMLE and other cells in vitro

It was found that Zeb1 constitutively expressed in HMLE cells could induce the EMT on a much shorter timescale than other known EMT-inducing factors. In an attempt to replicate the prior results, it was observed that the inducing the transition into a mesenchymal population of cells by the previously known factors could in part be explained by selection of a pre-existing mesenchymal subpopulation within the HMLE bulk population. Mesenchymal cells are more resistant to chemical and genotoxic insult than their epithelial counterparts, and are prone to arise in situations where there is a general survival selection pressure(Gupta et al., 2009),(Li et al., 2008). Thus, any study of EMT-induction must attempt to distinguish "EMT-by-differentiation" and "EMT-by-selection". In the case of Zeb1, it is straightforward, as no cell death is observed during the process of introducing the exogene, and the molecular markers and morphological characteristics of an EMT arise well before the selective outgrowth of a minor subpopulation can take place.

HMLE cells which undergo EMT grow more slowly, and there was difficulty in maintaining a pure mesenchymal population of cells, as a small proportion of cells which have escaped EMT for some reason or another would always eventually overwhelm the mesenchymal cells over the course of a few weeks. To counter this problem, single-cell clones of HMLE containing doxycycline-inducible Zeb1 expression constructs were derived. In this system, the addition of doxycycline results in the rapid induction of the production of Zeb1, and since the exogene is integrated into the same genomic site across the entire population, the response of the cells should be reasonably uniform. The cells in constant exposure to doxycycline remained stably mesenchymal for extended culture periods lasting more than a month. This advance solved the problem of the minor epithelial cell population overtaking the bulk, and simultaneously eliminated the possibility that Zeb1 induction only produced a transient EMT which is followed by a Mesenchymal-to-Epithelial Transition (MET) regardless of the continued expression of Zeb1.

The single-cell clone inducible system for the first time allowed some new experiments to be performed to study the effects of Zeb1. One of the early findings was that after the cells had undergone EMT, they can remain stably mesenchymal in the absence of doxycycline and the exogenous Zeb1. It was later found that exposure to doxycycline for as little as 24 hours committed the entire cell population to undergo an EMT (Fig. 1). These properties have never been demonstrated so vividly with any other EMT-inducing factor. Most EMT-inducing factors take weeks to produce a stable phenotype, giving amply time for the EMT-by-selection scenario to take place. It indicates that Zeb1 could be a crucial switch directly impinging on the decision process of whether to undergo an EMT or not.

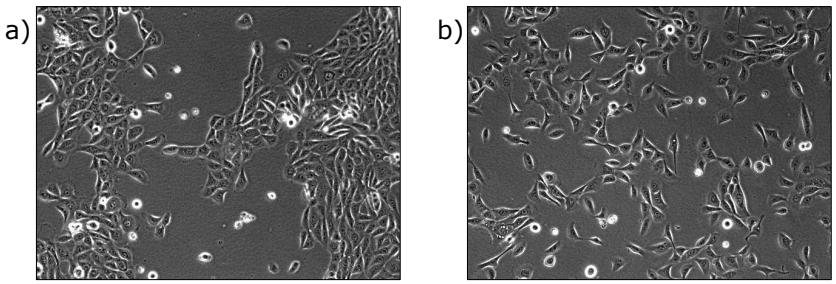

Figure 1. Cell morphology of single cell clones containing doxycycline-inducible Zeb1 expression constructs. Cell before exposure to doxycycline (a) and cells after 24 hours exposure to doxycycline, followed by two cell passages (b)

The cells that have stably turned mesenchymal are themselves a useful resource. Since they are no longer actively being perturbed by the exogene, they serve as a perfect reagent for determining what is required to induce an MET. This complete transition from epithelial to mesenchymal and back to epithelial again has never been robustly observed. Yet if EMT plays a role in metastasis, MET must occur, too, since the metastases of carcinomas tend to look morphologically similar to their parents, meaning they are epithelial rather than mesenchymal. Experiments are underway to introduce shRNA against Zeb1, Snail, and Twist into these cells, as well as reagents to interfere with various signaling pathways of the cells, in the hope of demonstrating a robust MET phenotype.

Since Zeb1 is a transcription factor, one plausible avenue to study its molecular biology is to perform chromatin immunoprecipitation followed by deep sequencing (ChIP-SEQ) on cells overexpressing Zeb1. This has been hindered by the problems in obtaining a good antibody against Zeb1. Some commercially available antibodies were tested and found not to detect Zeb1 at all, while others could detect Zeb1 but gave high background. Eventually, a polyclonal antibody against Zeb1 was found with reasonable selectivity, and in a collaboration with another member of the lab with ChIP-SEQ experience, the experiment was performed. Data analysis is underway.

The alternative approach to studying the genes directly targeted by Zeb1 is to perform a short-timecourse gene expression profiling experiment. With the single-cell clones, this becomes possible. The technical challenge is to obtain a strong signal from the immediate downstream genes while excluding the signal from secondary downstream effects. A known secondary downstream effect is the upregulation of endogenous Zeb1, which is due to the downregulation of the microRNA miR-200C (which targets Zeb1)(Burk et al., 2008), which in turn is due to the expression of exogenous Zeb1 (which targets and directly represses miR-200C expression)(Burk et al., 2008). This effect is observed 24 hours after doxycycline is added (Fig. 2), meaning this timepoint cannot be used for sampling the immediate downstream gene effects. At the optimal timepoint for the experiment, there would be a strong signal of miR-200C levels going down, but no significant indication of an increase in endogenous Zeb1 levels. Work is ongoing to optimize the system to maximize the immediate early signal and obtain the best timepoint. The window for the optimal timepoint is currently under 18 hours, but can probably be further improved.

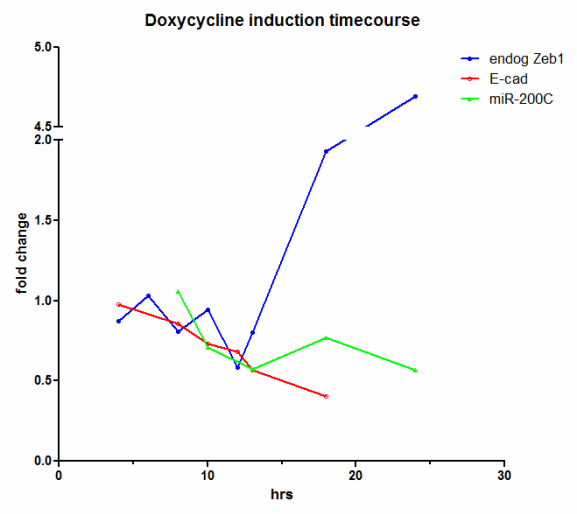

Figure 2. Preliminary results of levels of endogenous Zeb1, E-cadherin and miR-200C transcript levels as measured by realtime PCR. Time is measure as hours after addition of doxycycline, and fold change is being compared to uninduced cells

4. Demonstrate that key components of the EMT can profoundly influence the ability of tumorigenic cells to metastasize in vivo

Not yet underway. Tumorigenic equivalents of the doxycycline-inducible single-cell clones are being generated for this experiment, as it has been decided that polyclonally infected cells are not sufficiently uniform to give reliable results.

5. Correlate the activity of key components of the EMT with metastasis of human breast cancers and other cancers, using laser capture microdissected tumor samples

Not yet underway.

6. Develop a usable signature of EMT that can be used as a predictor of a primary tumor's tendency to metastasize

Not yet underway

Key Research Accomplishments

- Generated EMT gene signature from microarray data
- Identified Zeb1 as key candidate transcription factor mediating EMT
- Generated an inducible system for turning on the expression of Zeb1 in a clonal population of cells
- Established that Zeb1 is able to induce a true EMT-by-differentiation, compared with other factors which may partly accomplish an apparent EMT by selection
- Established that exogenous Zeb1 is only transiently required for the triggering of EMT, and is not required for the maintenance of the mesenchymal phenotype

Reportable Outcomes

- EMT gene signature
- Single-cell clones of Human Mammary Epithelial Cells expressing Zeb1 under control of a doxycycline-inducible promoter, and relevant control lines
- Stably mesenchymal single-cell clones of Human Mammary Epithelial Cells, not expressing any exogenous product in the absence of doxycycline.

Conclusion

Most work on EMT-inducing factors treat them individually and do not consider how they might work together or how they might differ qualitatively in what they actually do. The results obtained so far in this study indicate that Zeb1 is more important in the EMT than is currently appreciated, and may be more directly associated with the EMT than the other known factors. This makes it all the more important to identify exactly how Zeb1 is triggering the EMT.

The general assumption is that Zeb1 acts to repress genes, although there is no biochemical or molecular basis to show that this is its sole mechanism of action. There is no known pathway linking any EMT-inducing factor and the upregulation of mesenchymal genes such as vimentin and N-cadherin. An unbiased approach to studying the genes directly controlled by Zeb1 may reveal such a link and further our understanding of the mechanism of the EMT.

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